REMARKS

Claims 9, 13 and 15-17 presently appear in this case. No claims have been allowed. The official action of April 20, 2005, has now been carefully studied. Reconsideration and allowance are hereby respectfully urged.

Briefly, the present invention relates to novel isolated polypeptides that have their expression in neural cells modulated when such neural cells are subjected to neurotoxic stress, and antibodies thereto.

The interview between the undersigned attorney and Examiner Kim on July 22, 2005, is hereby gratefully acknowledged. In the interview, applicants agreed to narrow the claim so as to avoid as many issues as possible in an attempt to place the case into condition for allowance. Applicants are essentially relying only on the diagnostic utility, and so the issues about apoptotic utility for analogs is avoided at this time. The examiner agreed that the proposed amendments would appear to overcome the written description rejection except for the percent identity issue. The examiner stated that he would ask guidance from the Quality Matrix with respect to this remaining issue.

Claims 9 and 13-22 have been rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. The examiner states that the

examples in the written description guidelines examiner training materials are inapplicable because claim 13 is drawn to an isolated polypeptide whose activity is unknown. The examiner states that the present specification discloses a single species without describing any function associated therewith. The examiner states that applicants have not established whether the claimed polypeptide actually "induces" apoptosis. The examiner states that absent such clear functional description of the claimed protein, one skilled in the art would not readily recognize that applicants were in possession of the genus claimed. With respect to hybridization under stringent conditions, the examiner states that the instant specification and the declaration failed to give evidence to support that applicants were in possession of nucleic acids that hybridize to SEQ ID NO:3 under stringent conditions nor the proteins encoded by such acids that modulated and induced apoptosis. This rejection is respectfully traversed.

First of all, for the record, applicants disagree with the examiner's position that no utility relating to the modulation and inducement of apoptosis has been shown to exist for the present compounds. In this regard, the examiner's attention is invited to the attached publication of Budenov et al, "Identification of a novel stress-responsive gene Hi95

involved in regulation of cell viability," Oncogene 21:6017-6031 (2002).

Nevertheless, in order to expedite allowance of this case, the claim has now been amended to relate only to the naturally-occurring polynucleotide and naturally-occurring variants thereof that have a very high degree of homology. The claims no longer read on non-naturally occurring analogs. Regardless of whether or not the examiner agrees that the protein of the present invention induces apoptosis, there can be no disagreement that the mRNA encoding this protein is modulated significantly upward after the cells have been subjected to hypoxia. Indeed, that is how the gene was discovered. The diagnostic methods of the present invention are discussed in detail beginning at section VI of the present specification at page 40 through page 54. The Chumakov Declaration of record establishes that not only is the DNA modulated upward after the cells have been subject to hypoxia, but also the protein encoded thereby. Accordingly, the polypeptides being claimed in claim 13 have a diagnostic utility in order to determine that cells that produce such protein in augmented amounts have a likelihood of having been recently subjected to hypoxia.

Clearly, not only the naturally-occurring polynucleotide comprising SEQ ID NO:3 would have this property

for the purpose of the diagnostic utility, but also naturally-occurring allelic variations thereof. Claim 13(ii) has been amended to require that the naturally-occurring polynucleotide have at least 95% identity with the polynucleotide comprising SEQ ID NO:3, and claim 13(iii), requires that the naturally-occurring polynucleotide be capable of hybridizing under highly stringent conditions to the polynucleotide comprising SEQ ID NO:3. The claims require that such naturally occurring variants, in naturally occurring neural cells, have their expression modulated when the cells are subjected to neurotoxic stress.

It is believed that the presently-amended claims are no longer subject to the grounds of rejection set forth in the present written description requirement. The 95% identity requirement and the highly stringent hybridization conditions requirement should satisfy the written description guidelines for the same reason as Example 14 of the examiner training materials was found to satisfy the requirements. In other words, the claim requires the properties needed for a diagnostic assay. As long as the naturally occurring variant has these properties and the requisite degree of sequence identity, it has utility and is within the scope of the claims. Reconsideration and withdrawal of this rejection are, therefore, respectfully urged.

It is submitted that all of the claims now present in the case clearly define over the references of record and fully comply with 35 U.S.C. §112. Reconsideration and allowance are, therefore, earnestly solicited.

Respectfully submitted,

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Identification of a novel stress-responsive gene Hi95 involved in regulation of cell viability

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cDNA microarray hybridization was used in an attempt to identify novel genes participating in cellular responses to prolonged hypoxia. One of the identified novel genes, designated Hi95 shared significant homology to a p53-regulated GADD family member PA26. In addition to its induction in response to prolonged hypoxia, the increased Hi95 transcription was observed following DNA damage or oxidative stress, but not following hyperthermia or serum starvation. Whereas induction of Hi95 by prolonged hypoxia or by oxidative stress is most likely p53-independent, its induction in response to DNA damaging treatments (γ- or UV-irradiation, or doxorubicin) occurs in a p53-dependent manner. Overexpression of Hi95 full-length cDNA was found toxic for many types of cultured cells directly leading either to their apoptotic death or to sensitization to serum starvation and DNA damaging treatments. Unexpectedly, conditional overexpression of the Hi95 cDNA in MCF7-tet-off cells resulted in their protection against cell death induced by hypoxia/glucose deprivation or H_2O_2. Thus, Hi95 gene seems to be involved in complex regulation of cell viability in response to different stress conditions.
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Keywords: hypoxia; p53-responsive gene; cell viability; stress response

Introduction

Normal function of organs and tissues in the organism requires an appropriate oxygen supply. Local oxygen deprivation (hypoxia) is usually caused by vascular deficiency and obstruction and leads to such common pathological conditions as stroke and myocardial infarction. Maintenance of oxygen homeostasis in the organism is controlled by oxygen-sensing mechanisms that result in cell-specific induction of certain genes aimed to compensate for oxygen deficiency. As a consequence, increased production of erythropoietin, VEGF, iNOS and glycolytic enzymes stimulates production of red blood cells, helps to build collateral vessels, achieves local vasodilation, and changes carbohydrate metabolism, respectively, to ensure appropriate energy balance (for review see Guillemin and Krasnow, 1997). However, in severe and prolonged hypoxia, such adaptive responses might fail to secure normal function and integrity of tissue. In this case, induction of genes that control cell death might be important to ensure prevalence of apoptosis over necrosis and subsequent healing of affected organs. Revealing the nature of genes that control cell viability in response to prolonged hypoxia is essential for developing novel drugs targeted to reduce the toxic effects of prolonged oxygen deprivation to the most sensitive cellular components of the tissue. In an attempt to identify novel genes involved in the regulation of cell death in response to prolonged hypoxia, we chose to employ a microarray hybridization technique to compare gene expression profiles in human glioblastoma A172 cells maintained under normoxic conditions, and following their exposure to hypoxia for two different time intervals, short (4 h) and prolonged (16 h). In accordance with the assumption that proapoptotic genes should be upregulated after prolonged rather than after short hypoxic treatment, we have concentrated on genes induced solely at the 16 h time point. One such gene, further designated Hi95, was chosen for further characterization. By microarray analysis, it displayed ~3.5-fold upregulation under hypoxic conditions. The choice of the gene was further reinforced by its strong homology to p53-responsive gene PA26 (Velasco-Miguel *et al.*, 1999), which has been added to the expanding list of growth arrest and DNA damage (GADD) genes (Fornace *et al.*, 1989). The GADD genes that are induced in response to genotoxic treatments encode functionally diverse protein products participating in a range of

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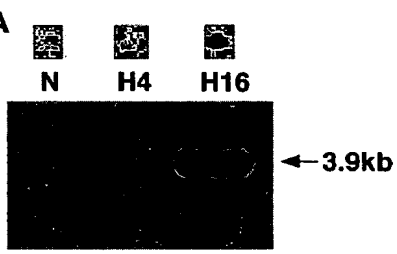
adaptive or detrimental responses to various stresses (Fornace, 1992; Fornace *et al.*, 1992; Smith and Fornace, 1996). We found that in addition to prolonged hypoxia, the Hi95 gene is also responsive to DNA damage and oxidative stress, hence, like its homolog PA26, closely resembling the GADD genes. By testing the biological consequences of either transient constitutive or conditional overexpression of Hi95 gene in different cell lines under various stresses, we obtained convincing evidence suggesting an important role of the Hi95 gene in cell survival.

Results

Cloning and sequence analysis of a novel gene Hi95

In search for hypoxia-responsive genes, we have employed a microarray-based analysis of genes differentially expressed in human glioma cell line A172 cultured in normal conditions or following 4 and 16 h exposure to hypoxia. One of the previously non-described gene induced ~3.5-fold by 16 but not by 4 h hypoxia was selected for further study (Figure 1a). We have designated it Hi95 (for Hypoxia induced gene #95). The pattern of its hypoxia-regulated expression was confirmed by Northern blotting (Figure 1a) using A172-derived mRNA. It turned out that Hi95 is transcribed as a single mRNA species of 3.9 kb. We next constructed a cDNA library from A172 cells grown for 16 h in hypoxic conditions and screened it with the Hi95-specific probe originated from its 3′UTR fragment printed on the microarray. A λ phage clone with a 3534 bp Hi95 cDNA insert has been isolated. Nucleotide sequencing revealed that the cDNA contains a single open reading frame encompassing nucleotides 322–1761 with a coding capacity for a putative protein of 480 amino acids (Figure 1b). *In vitro* translation of this cDNA gave rise to a protein of ~60 kDa (not shown). The nucleotide and protein sequence of Hi95 was compared to public sequence databases and was found homologous but not identical to a previously described human p53-regulated gene PA26 (Velasco-Miguel *et al.*, 1999), suggesting the existence of a novel protein family within the mammalian genome.



B

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CCT TCC CAG GCC CCC GAG GCC GTT CGC CGT TCC CGA AGC CCG ACT    135
GGG GGA AGA GTC CAG CAC CAA AGC GGC CGT TCT CGG ATT CCG GAG    180
CGT TCT GGA GCC CCG AGA GAC GCC CCG GGG TTC TAG AAG CTC CCC    225
GGC GGC GCC CAG TCC CGG CTT CAT TCG GGC GTC CCT CCG AAA CCC    270
ACT CGG GTG CAC GGG TCG TCG GCG AGC CGC GAC CGG GTC CTG GCG    315
CGC ACC ATG ATC GTG GCG GAC TCC GAG TGC CGC GCA GAG CTC AAG    360
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GGA GAG GAG CAG AGG GAG AGC CGG GCT CGG CGA GGC CCT CGA GGG    450
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CCC AGC GCC TTC ATC CCC GTG GAG GAG GTC CTT CGG GAG GGG GCT    495
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GCT GCC GCC CGC CAT CAG TGT TCT TAC CTG GTA GGC TCC CAC ATG    720
  A   A   A   R   H   Q   C   S   Y   L   V   G   S   H   M    133
GCC GAG TTT CTG CAG ACT GGT GGT GAC CCT GAG TGG CTG CTG GGC    765
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TGG GAA GAC CAT GGC TAC TCG CTG ATC CAG CGG CTT TAC CCT GAG   1395
  W   E   D   H   G   Y   S   L   I   Q   R   L   Y   P   E    358
GGT GGG CAG CTG CTG GAT GAG AAG TTC CAG GCA GCC TAT AGC CTC   1440
  G   G   Q   L   L   D   E   K   F   Q   A   A   Y   S   L    373
ACC TAC AAT ACC ATC GCC ATG CAC AGT GGT GTG GAC ACC TCC GTG   1485
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ATG CAA GCC GCT CTG CTG TAC GCC CTC CGT GCC ATC ACC CGC TAC   1755
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  M   T                                                      480
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CAT GCC CAG GCT CCA CAG GCT GCA CTG GGC TTG TGT GTG ATG TGC   1890
AGT CCC GAA GCC ACA CCC TCC CTT TTC CTC ACT GGA ATG GAC AGT   1935
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AAG AGC ACT TGG AGA TCC TAA GGG ACC ACA CCC TTC CTC CTT CCC   2025
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GTC CCT GGC TGG CCC CCG GGG GAG AGG GCC AAA TGC CTC CCG GAC   2160
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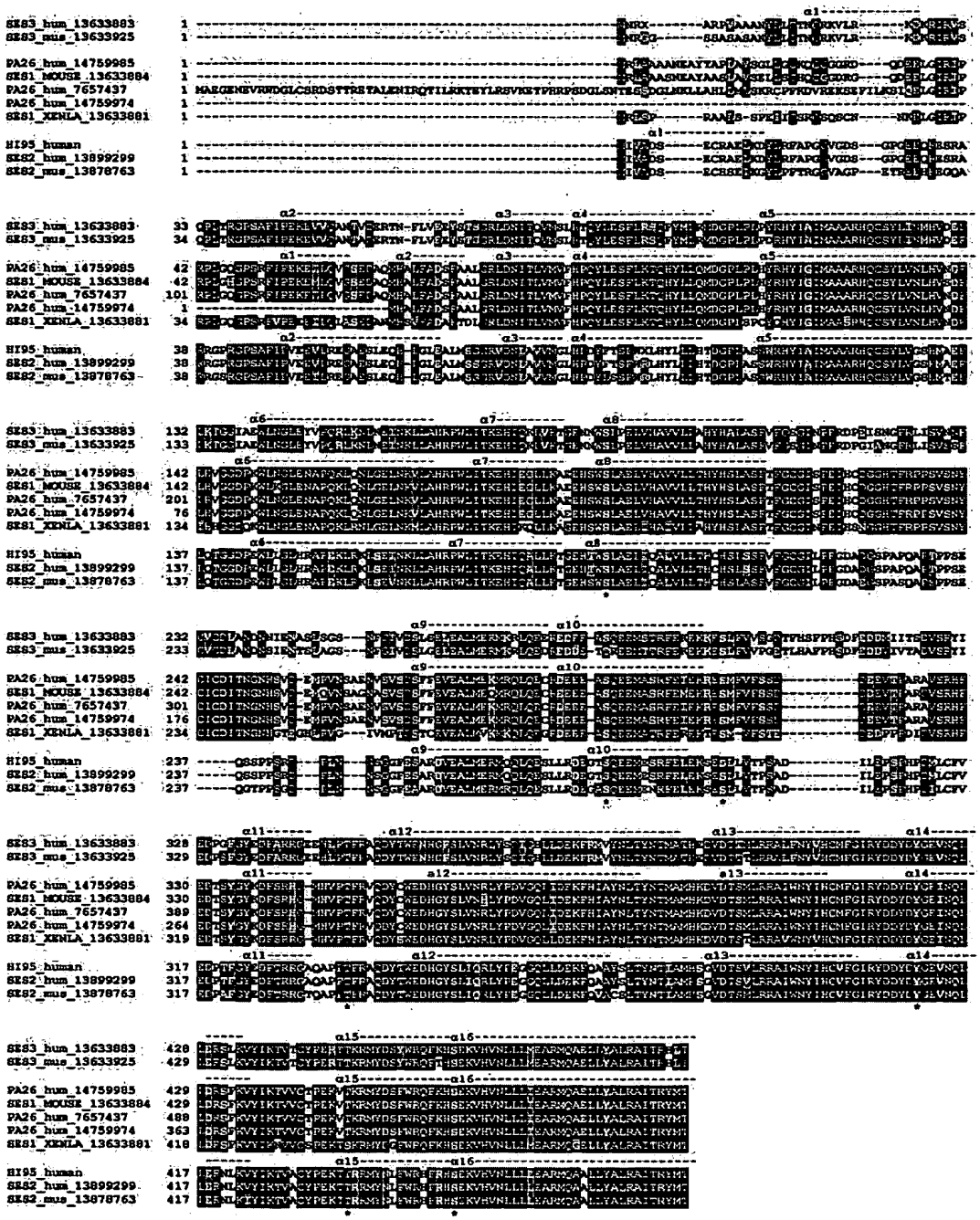

Figure 1 Regulation of Hi95 gene expression by hypoxia. RNA was extracted from A172 glioblastoma cells maintained under normoxia (N) or after 4 h (H4) and 16 h (H16) of exposure to hypoxia. This RNA was either used for hybridization to microarray (upper panel: images of Hi95-specific hybridization signal) or for Northern blot analysis with Hi95-specific probe (lower panel). **(b)** Nucleotides and putative amino acids sequence of cloned Hi95 cDNA. Polyadenylation signal is underlined. **(c)** Alignment of sestrin family of proteins. The alignment was performed with the aid of BioEdit Sequence alignment editor. The positions of α-helices are indicated as dashed red lines over the alignment. Predicted cAMP-dependent kinase, CK2, PKC and tyrosing phosphorylation sites are shaded with pink, green, blue, and red, respectively. Phosphorylation sites conserved among all three types of sestrins and among different species are labeled with asterisks below the alignment. Multiple tyrosine residues within the C-terminal portions of sestrin proteins are shown with yellow 'Y' letters

PA26 is transcribed in a form of three alternatively spliced messages which share a common set of 3′ exons while possessing different first exons and hence, different transcription initiation points. Among the three transcripts, a constitutive 4 kb and a p53-responsive 2.6 kb mRNA are the predominant ones. They are expressed in various quantities in many human tissues. Northern blot analysis using a Hi95 cDNA probe that excluded the possibility of cross-hybridization with PA26 revealed only a single 3.9 kb mRNA expressed at low to moderate levels in most human tissues tested (not shown). When this paper was in preparation, the repeated database search revealed several recent entries for hypothetical protein sequences that were submitted under a family name of sestrins, whereas the gene designated as sestrin 1 was identical to PA26, sestrin 2 to Hi95, and sestrin 3 still remains a hypothetical protein. The multiple sequence alignment of mouse and human sestrin protein family members is shown in Figure 1c. A recently cloned *X. Laevis* ortholog of sestrin 1 (PA26) (Hikasa and Taira, 2001) was also added to the alignment. The gene family is well conserved in evolution since the *C. elegans* and *Drosophila* orthologs were predicted from the corresponding genomes. They display the highest similarity to sestrin 1 (not shown). All three types of sestrin family members are predicted to be compact globular domain proteins (GLOBE, http://cubic.bioc.columbia.edu/predictprotein) composed predominantly of α-helical regions (PHD, http://cubic.bioc.columbia.edu/predictprotein). Interestingly, the regions most conserved among the sestrin proteins fall within the α-helices. Roughly, there are three highly conserved helical regions (helices α3–α8, α9–α10, and α11–α16),

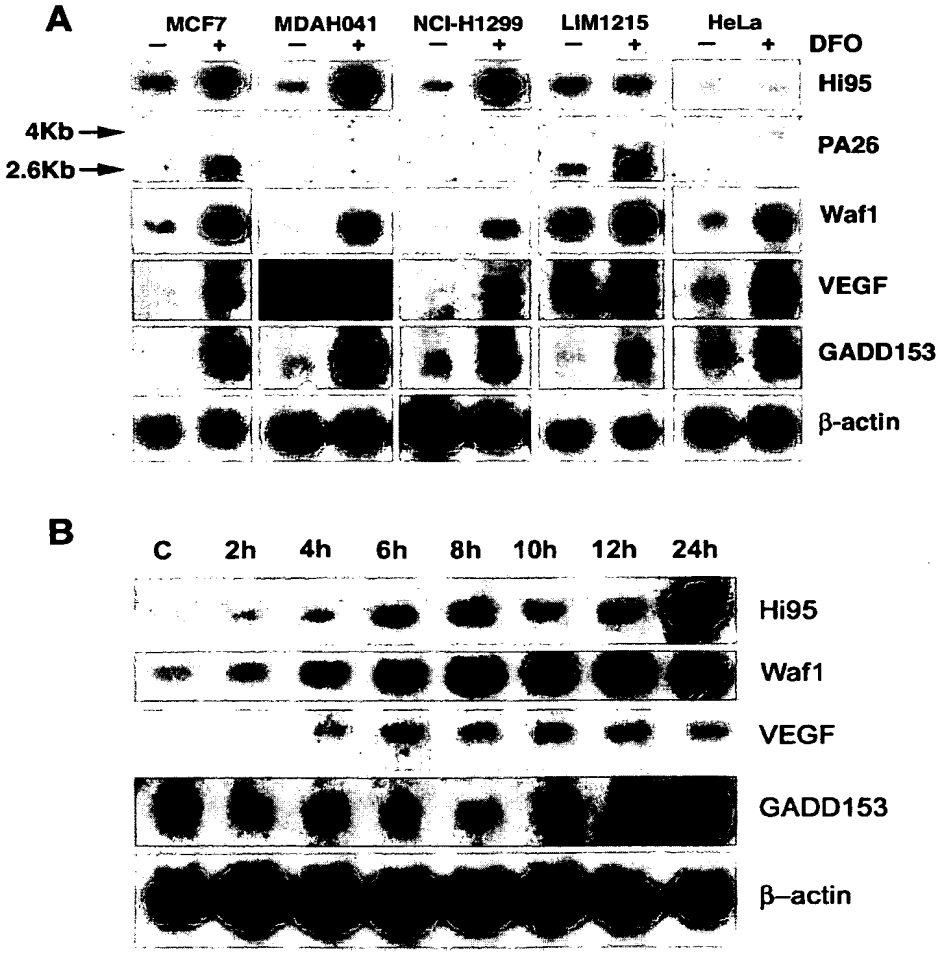

Figure 2 (**a**) Northern blot analysis of Hi95 expression in different cell lines following 24 h treatment with hypoxia-mimicking agent, DFO. Ten μg of total RNA were loaded in each slot. RNA was transferred to Hybond N membranes and hybridized to the indicate probes. 2.6 and 4 kb PA26-specific mRNAs are indicated by arrows. (**b**) Kinetics of accumulation of Hi95-specific transcript following the DFO treatment MCF7 cells. Ten μg of RNA extracted either from the non-treated control cells (C) or from the cells treated for different intervals of time (from 2 to 24 h) with DFO were loaded into each slot, electrophoresed, blotted and hybridized to the indicated gene-specific probes. In **a** and **b**, the β-actin probe served as RNA-loading control

separated by less conserved hinge sequences. The region of high conservation corresponds to the common exon set present in all three spliced versions of PA26 (sestrin 1). The N-terminal sequences of sestrins are less conserved, although it is clear that both Hi95 (sestrin 2) and sestrin 3 most closely resemble the major PA26 protein encoded by T2 2.6 kb mRNA. The ProSite (http://www.expasy.ch/prosite) analysis of sestrins revealed that they all contain several potential serine-threonine and tyrosine phosphorylation sites, most of them again being located within the α-helices. Thus, all three types of sestrins contain two conserved potential CK2 phosphorylation sites in helices α8 and α10, three PKC phosphorylation sites in helices α11, α15 and α16, one cAMP/cGMP-dependent protein kinase phosphorylation site in helix α10, and one tyrosine phosphorylation site within helix α14. It is worth noting that helices α11–α16 contain 12–13 additional tyrosine residues that, although not predicted to be the sites of phosphorylation, may probably serve as such, potentially implicating tyrosine-based signal transduction as a mode of sestrin regulation.

Hi95 expression is induced by hypoxia both in vitro *and* in vivo *in various cell types*

To study the mode of induction of Hi95 expression under hypoxic conditions, we tested accumulation of its mRNA in several cell lines of different origin (see Materials and methods) subjected to treatment with hypoxia-mimicking drug deferoxamine mesylate (DFO). We were also interested in comparing the hypoxia response of Hi95 with that of known hypoxia-responsive genes such as p21-Waf1 (Long *et al*., 1997), VEGF (Carmeliet *et al*., 1998), or GADD153 (Price and Calderwood, 1992), as well as to a closely homologous gene, PA26. For this, Northern blot containing mRNA from different control and DFO-treated human primary cell cultures and cell lines, was consequently hybridized to the corresponding cDNA probes. The choice of Hi95 and PA26 specific probes excluded cross-hybridization. The representative results of this analysis are shown in Figure 2a. We found that Hi95 expression was upregulated by DFO treatment in all analysed cell lines with the exception of HeLa and LIM1215. In HeLa cells, Hi95 transcript has hardly detected, while in LIM1215 the gene was expressed at a level comparable with i.e. MCF7 cells. All three co-analysed control hypoxia-regulated genes, p21-Waf1, VEGF and GADD153, were up-regulated by DFO in all cell lines tested without any exception, while PA26 was responsive only in cells that retained functional wild type p53, e.g. in MCF7 and LIM1215. As expected, only a 2.6 kb PA26 transcript displayed inducible behavior, whereas the levels of 4 kb transcript remained constant. We next tested whether the slow kinetics of Hi95 RNA accumulation in response to hypoxia is reproduced in addition to A172 cell lines. Indeed, the kinetics of the Hi95 response to hypoxia in MCF7 cells was also relatively delayed, reaching maximal expression levels between 12–24 h of DFO treatment and mostly resembling that of the GADD153. In contrast, p21-Waf1 and VEGF genes were induced earlier, reaching a maximum by 8–10 h (Figure 2b).

To verify whether Hi95 gene is induced by the lack of oxygen also *in vivo*, two hypoxia-associated pathological conditions were analysed for Hi95 expression by *in situ* hybridization. Specifically, Hi95 expression was assessed in hypoxic region of tumors (chronic hypoxia) and in an acute hypoxia model of stroke created in rats by permanent middle cerebral artery occlusion (MCAO). In both cases, the VEGF-derived probe was employed to delineate the hypoxic regions within the tissue. Indeed, in C6 rat gliomas grown subcutaneously in nude mice, significant levels of Hi95 expression were detected in VEGF-positive regions consistent with hypoxia-dependent regulation (Figure 3). Being undetectable in untreated rat brain, expression of Hi95 was observed in the MCAO model

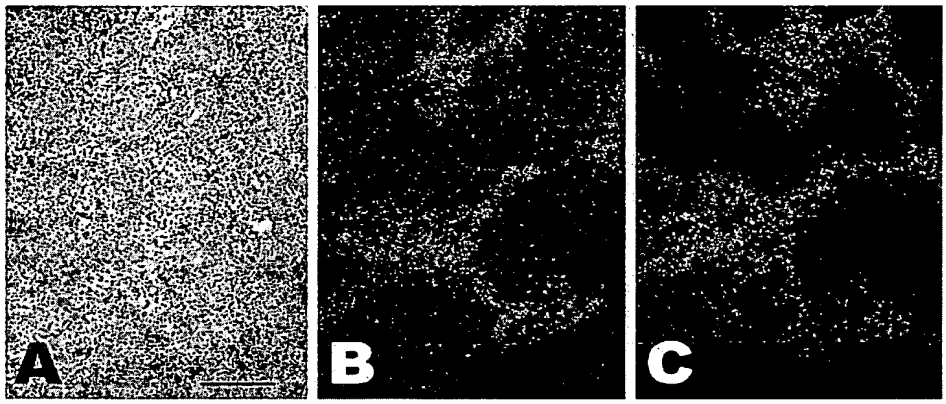

Figure 3 Expression of Hi95 gene in C6 rat glioma-derived tumor grown subcutaneously in nude mouse. Hi95-expressing cells (**a**,**b**) are localized to hypoxic/ischemic areas of the tumor delineated by hybridization of the parallel section to VEGF-specific probe (C). (**a**) Bright-field micrographs (**b**,**c**) Dark-field micrographs. Hybridization signal appears as shining dots in the dark-field images. Magnification × 20

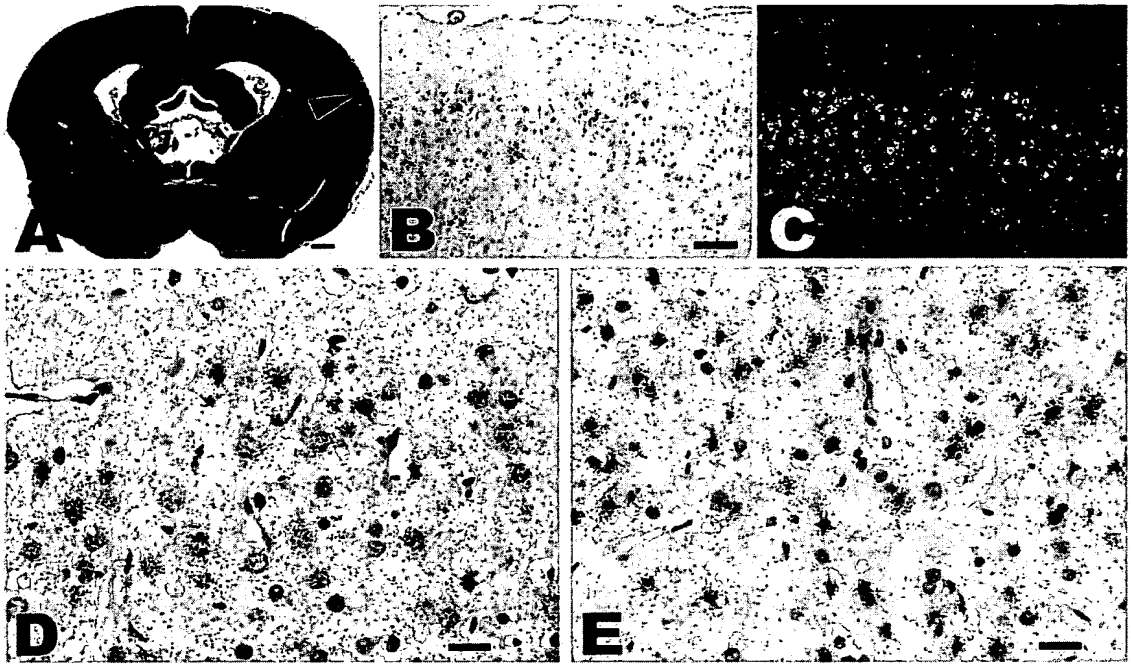

Figure 4 Expression of Hi95 gene in rat brain subjected to permanent middle cerebral artery occlusion (MCAO). (**a**–**d**) Analysis of Hi95 expression in rat brains following 12 h post MCAO. (**a**) Low-power microphotograph of coronal brain section hybridized to Hi95-specific probe. The higher magnification images shown in **b**–**d** are located within the peri-infarct area pointed to by the arrowhead in (**a**). (**e**) High-power microphotograph of periinfarct/perinecrotic area in rat brain fixed at 24 h post MCAO. (**b**, **d**, **e**) Bright-field microphotographs. (**c**) A dark-field microphotograph. Hi95-expressing neurons are revealed by hybridization signal appearing as black or white spots in the bright-field and dark-field images, respectively. Scale bars: **a** – 1 mm; **b**, **c** – 100 μm; **d**, **e** – 25 μm

in the TUNEL-positive (not shown) peri-infarct areas (Figure 4b–e). Interestingly, similar to *in vitro* studies, the kinetics of Hi95 upregulation was delayed, peaking at 12–24 h. Whereas at 12 h post-stroke, the Hi95 expressing neurons displayed relatively normal morphology (Figure 4d), at 24 h most of them appeared shrunken and contained dense nuclei (Figure 4e), consistent with the potential detrimental effect of Hi95 expression. Thus, we have concluded that Hi95 is induced by hypoxia both *in vitro* and *in vivo* in various cell types and with the same slow kinetics.

Hi95 is upregulated by hypoxia in a p53-independent manner but displays p53-dependent induction in response to DNA damage

High sequence similarity between Hi95 and PA26 suggested also their possible functional homology. Therefore, we decided to test whether expression responses of Hi95 resemble those of GADD genes, and whether its regulation might depend on the functionality of p53 pathway. To test this, several human cell lines were treated either with doxorubicin, or UV or γ-irradiation. Following the treatments (see Materials and methods), RNA was extracted and analysed by Northern blotting using probes specific for Hi95, PA26 and p21-Waf1 genes. Representative examples of the obtained results are shown in Figure 5a (γ-irradiation treatments are not shown). Expression of the Hi95 transcript was found elevated in response to all types of DNA damaging treatment applied. However, unlike hypoxia, DNA damage-triggered upregulation was strictly dependent on the presence of functional p53. Thus, in p53-null cells like MDAH041 or NCI-H1299, none of the genotoxic treatments was capable of Hi95 induction, while its 3.9 kb transcript was clearly stimulated by DFO.

To further confirm the role of p53 in mediating Hi95 gene regulation, NCI-H1299-tet-off cells engineered to express wild-type p53 under the control of tetracycline-repressible promoter were employed. The NCI-H1299-p53 cells maintained either in the presence or in the absence of tetracycline were treated with either DFO, or doxorubicin, or UV, as described above, and their RNA was subsequently tested for Hi95, PA26 and p21-Waf1 expression by Northern blotting. As evident from Figure 5b, wild-type p53 expression was essential for both PA26 and Hi95 up-regulation in response to DNA-damaging agents, while in the case of DFO treatment p53 was necessary only for stimulation of PA26. Functional p53 was unnecessary for induction of Hi95 by oxidative stress (H_2O_2). The gene appeared inert to conditions of heat shock and serum starvation (not shown).

To estimate the kinetics of Hi95 transcriptional response to DNA damage and its relationship to

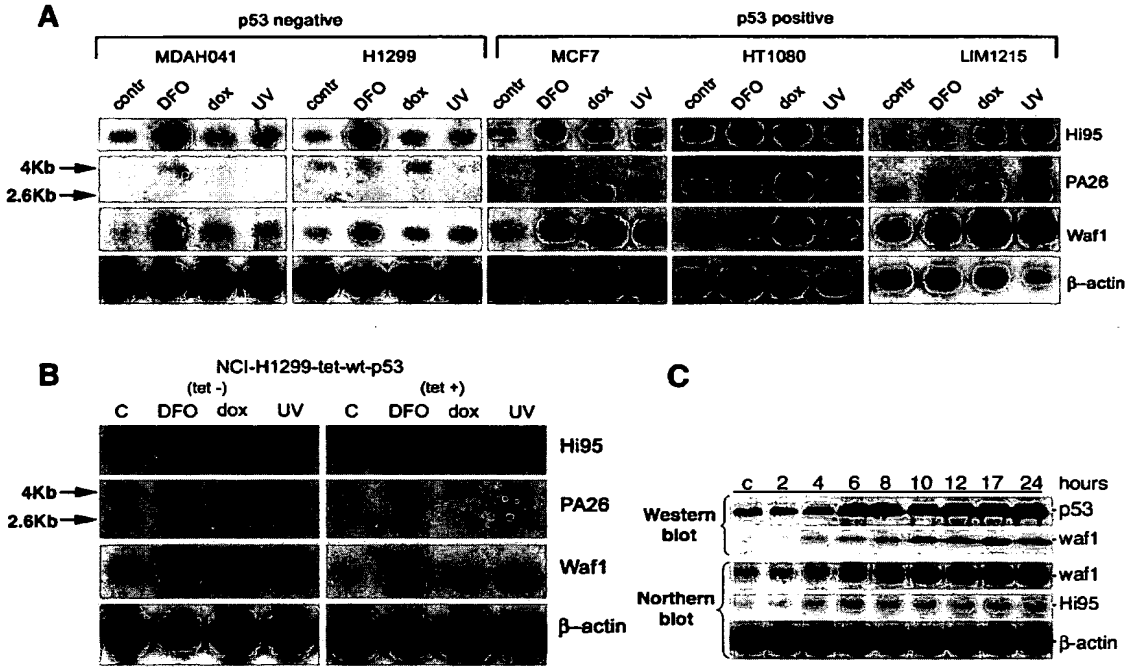

Figure 5 Northern blot analysis of Hi95 expression in response to DNA-damaging treatments. The cell lines names and their p53 status is indicated over the Northern blots images. Positions of 2.6 and 4 kb PA26-specific transcripts is shown with arrows. (**b**) Northern blot analysis of DNA damage-dependent Hi95 expression in p53-negative NCI-H1299 cells bearing tetracycline-repressible wild-type p53. The cells were incubated either in the presence (P53-negative, right panel) or in the absence (p53-positive, left panel) of tetracycline and were treated with DNA damaging agents as described in Materials and methods. Positions of 2.6 and 4 kb PA26-specific transcripts is shown with arrows. (**c**) Kinetics of Hi95 transactivation in MCF7 cells in response to DNA damage. MCF7-tet-off cells were treated with doxorubicin as described in Materials and methods. Protein and RNA were extracted following the indicated time intervals and analysed by Northern (lower panel) and Western blotting (upper panel). For immunoblotting, anti-p53 mouse monoclonal DO-1 and anti-waf1 mouse monoclonal F-5 antibodies (both Santa Cruz) were used. In **a** and **b**, DFO – deferoxamine mecylate, dox – doxorubicin, UV – ultraviolet irradiation (for details, see Materials and methods). In **a**, **b** and **c** 10 μg of total RNA from control non-treated and treated cells was loaded per slot, electrophoresed, blotted and hybridized to the indicated gene-specific probes. β-actin-specific probe served as RNA-loading control

DNA-damage-induced stabilization of p53, RNA and protein were extracted from doxorubicin-treated MCF7 cells at different time points from the beginning of the treatment. As shown in Figure 5c transactivation of Hi95 was first observed after 4 h coinciding in time with the initiation of transactivation of p21-waf1 and p53 stabilization.

Thus, from the above observations, it appears that Hi95, being structurally similar to GADD gene PA26, is also regulated in a manner similar to typical GADD genes.

Expression of Hi95 elicits apoptosis in recipient cells

Many of the GADD genes possess growth-arrest-promoting or pro-apoptotic properties (Fornace *et al.*, 1992; McCullough *et al.*, 2001; Sheikh *et al.*, 2000; Zhang *et al.*, 2001). The function of the Hi95 homolog, PA26, has not been yet defined. To elucidate the function of the newly cloned Hi95 gene, we have constructed an expression pcDNA3 vector containing Flag-tagged Hi95 open reading frame.

Potential cytotoxic activity of Hi95, was assessed by quantitation of Annexin V positive HEK293 apoptotic cells following transient transfection of Hi95 expressing construct, as well as in colony formation assays with Hi95-transfected MDAH041, NCI-H1299, MCF7, LIM1215 and HT1080 cells.

HEK293 cells were transiently co-transfected with the pcDNA3-EGFP and pcDNA3-Hi95 expression vectors. In control transfection, pcDNA3-Hi95 was substituted with the empty vector. Forty-eight hours following transfection, the cells were harvested and stained with Annexin V-FITC. The proportion of Annexin V positive cells was determined by fluorescent sorting separately into GFP-positive (transfected) and GFP-negative (non-transfected) cell populations (Figure 6a). It was found that, while only 7.8% of control transfected GFP-positive cells were stained with Annexin V, their proportion reached 43% in Hi95-transfected GFP-positive cells. The percentage of Annexin V stained cells was similarly low (2.7 and 0.5%, respectively) in both GFP-negative cell populations regardless of whether Hi95 or control vector was

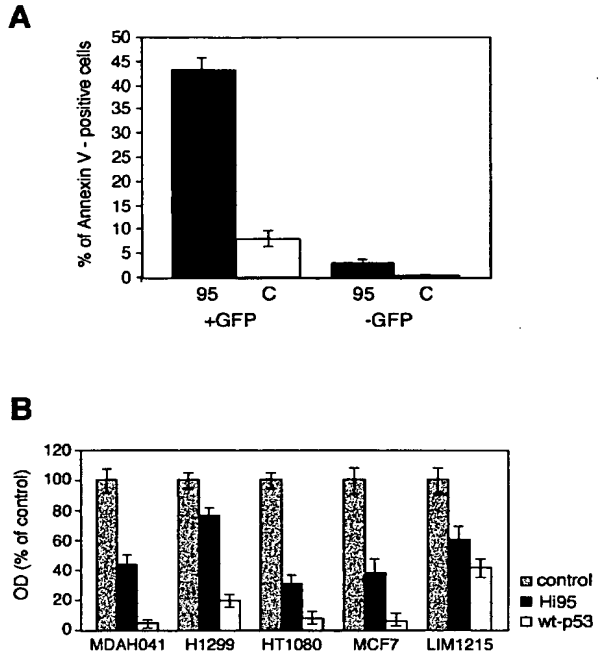

Figure 6 (a) Apoptosis of HEK293 cells transiently transfected with Hi95. Hi95 and EGFP expression vectors were co-transfected in HEK293 cells. Forty-eight hours post transfection, percentage (of total) of Annexin V-positive cells was measured in transfected (+GFP) and non-transfected (−GFP) cells within Hi95 (95) and control empty vector-transfected (C) cell populations. The presented results were obtained in three independent experiments. (b) Analysis of influence of Hi95 expression on colony growth of different cell types (colony formation assay). The indicated cells were transfected with pcDNA3-Hi95, or pcDNA3-wt-p53, or control pcDNA3 expression plasmids as described in Materials and methods. The G418 selection continued for ~12 days and was followed by cell staining with methylene blue dye. The dye was next extracted and measured at 540 nM. The experiment was repeated three times in duplicates

used for co-transfection. These results, which were repeatedly obtained in three independent experiments, strongly suggest that the observed apoptosis of HEK293 cells is Hi95-specific.

For the colony formation assay, 2×10^5 cells of MDAH041, NCI-H1299, HT1080, MCF7 and LIM1215 cell lines, were transfected with pcDNA3-Hi95 expression plasmid. The same amount of the empty vector and the vector expressing a classical growth suppressor p53 was used as negative and positive control, respectively. To normalize the efficiency of transfections, the EGFP-expressing plasmid was co-transfected in all the experiments, and the same number of EGFP-positive cells of each group was subjected to G418 selection. The resistant colonies were fixed on plates, stained with methylene blue and measured for optical density at 640 nM following methylene blue extraction (Figure 6b). In all of the cell lines tested, a variable extent of reduction in colony formation ability was observed following Hi95 transfection. The growth inhibitory effect of p53 expression in the same cells was however more significant. We concluded that exogenous overexpression of Hi95 is negatively influenced by cell growth in culture.

Inducible expression of Hi95 suppresses growth of MCF7 cells

To avoid the problem of cytotoxicity that rules out the preparation of cell lines with long-term expression of exogenous Hi95 we attempted construction of tetracycline-inducible cell lines. The N-terminal Flag-tagged full-length Hi95 open reading frame was subcloned under a tetracycline-repressible promoter into the pTet-Splice vector (BRL) and introduced into the MCF7-tet-off human breast carcinoma cell line expressing tetracycline-repressible transactivator (tTA) (Gossen and Bujard, 1992). An empty pTet-Splice vector was used as a negative control. Two independent hygromycin-resistant mass cultures, MCF7-tet-Hi95Flag1 and MCF7-tet-Hi95Flag2 (as well as a control mass culture), were tested for expression of Hi95 gene product by immunoblotting with anti-Flag antibodies at 24, 48, 72 and 96 h following tetracycline removal. Expression of Flag-tagged Hi95 proetin migrating in the expected region of approximately 60 kDa was detectable only after 72 h of induction (Figure 7a). Two closely migrating faint bands, approximately of the same size, were also detected by anti-Flag antibodies in the extract derived from MCF7-Hi95 cells maintained in the presence of tetracycline. However, these bands are likely to be non-specific since they were observed in control MCF7 cells as well.

As a first attempt to assess the influence of Hi95 expression on cell growth, the mass cultures of MCF7 cells with tetracycline-regulated Hi95 cDNA and the control MCF7 cells were grown either in the presence or in the absence of tetracycline for 1 week, with daily cell counting. No difference between control and Hi95 expressing MCF7-tet-off cells was found. Western blot analysis with anti-Flag antibodies carried out at the end of the experiment revealed the absence of the 60 kDa Hi95-specific band indicating the rapid elimination of the expressing cells from the culture (not shown). To avoid this problem, two individual cell clones, MCF-Hi95-2 and MCF-Hi95-20, were obtained from the mass culture by limited dilutions in the presence of tetracycline. We failed to confirm inducible expression of Hi95 protein (probably because its levels were under the detection limits), however its inducible RNA expression was confirmed (Figure 7b). Next, the effect of tetracycline withdrawal on cell growth was tested. Control and Hi95 expressing MCF7 cell clones were maintained for 72 h either in the presence or in the absence of tetracycline and the appearance of apoptotic Annexin V-positive cells was estimated by FACS analysis. As seen in Figure 7c, even in the presence of tetracycline, Hi95-transfected cultures constitutively contain more Annexin V positive cells compared to the control, potentially as a consequence of a certain leakiness of the system. Ninety-six hours following tetracycline removal (24 h after the moment

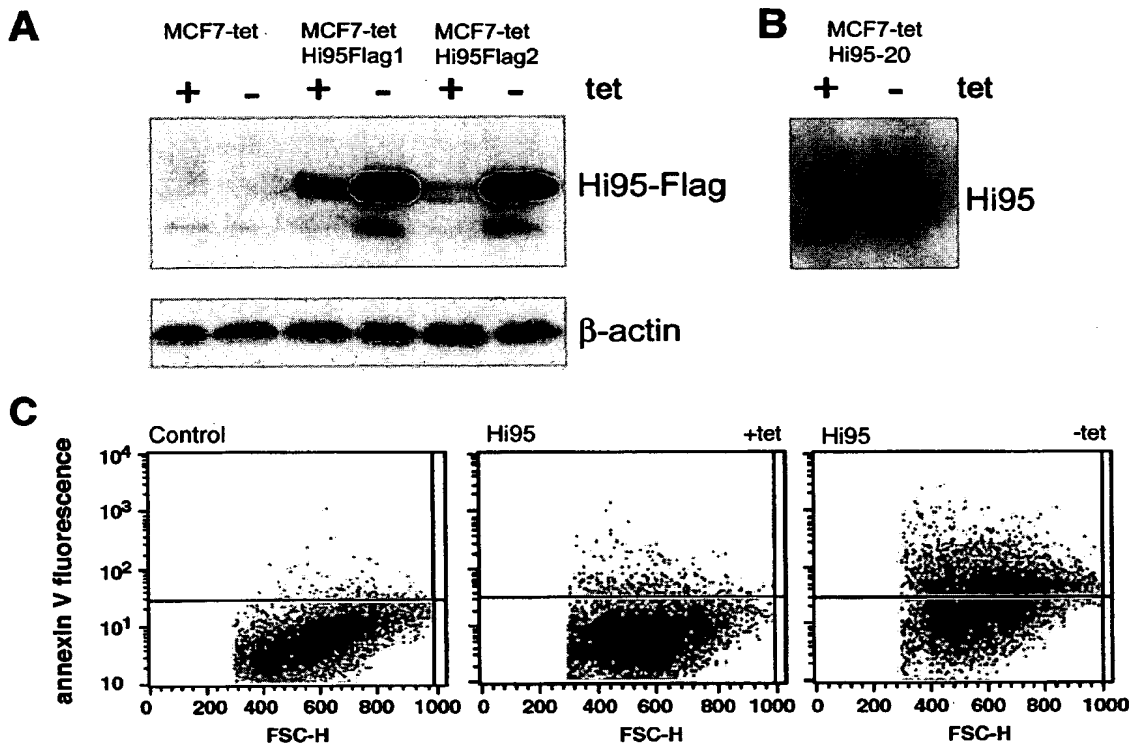

Figure 7 (a) Western blot analysis of inducible Hi95 expression in two independent MCF7-Tet-off polyclonal cell populations (Hi95Flag1 and Hi95Flag2) bearing plasmids with tetracycline-reglated Hi95-Flag. The cultures were maintained either in the medium containing 1 μg/ml tetracycline (+), or without tetracycline for 72 h before preparation of protein extracts (−). The membranes were hybridized to anti-Flag antibodies (Sigma). Position of Hi95-Flag protein is indicated. Immunoblotting with anti β-actin antibodies served as loading control. (b) Increased apoptosis of MCF-7-tet-off-Hi95 cells upon induction of Hi95 expression. Flow cytometry of Annexin V stained control and MCF-Hi95-2 cells in the presence (+tet) or in the absence (−tet) of tetracycline

Hi95 induction reached the detectable levels), the proportion of apoptotic cells has further increased in the Hi95-expressing but not in the control cells. The observed pro-apoptotic effect was less significant than in transient transfection experiments, probably due to the lower levels of Hi95 expression.

Inducible expression of Hi95 sensitized MCF7 cells to DNA damaging treatments and serum deprivation

We next tested whether the expression of Hi95 can influence cell sensitivity to various stressful conditions. For this, 1×10^4 of MCF-Hi95-2, MCF-Hi95-20 and MCF-Hi95-V (empty vector control) cells were plated in 6-well plates and maintained for 72 h either in the presence or in the absence of tetracycline. Then the cells were irradiated with UV for 1 min or treated with 100 ng/ml of doxorubicin for 4 days. At the end of each treatment, the medium was replaced with the fresh one, with or without tetracycline, and the cells were maintained for additional 24 h, fixed on plates and stained with methylene blue for visualization (Figure 8a). Under the described conditions, UV and doxorubicin had only a minor effect on the growth of both control and Hi95 cell clones maintained in the presence of tetracycline. However, in the absence of tetracycline both treatments suppressed the colony formation ability of Hi95 expressing cells by more than 90%. Inducible expression of Hi95 in MCF7 cells also made them more sensitive to serum deprivation, as measured by LDH release (Figure 8b), and neutral red uptake (not shown). These results indicate that under conditions where Hi95 itself has only marginal effect on cell growth or viability, it is able to sensitize the cells to some types of stress. Interestingly, treatment with 100 ng/ml of taxol revealed no difference between Hi95 inducible and control MCF7 clones (not shown).

Inducible expression of Hi95 protects MCF7 cells from ischemia and H_2O_2 treatment

Since Hi95 was found to be a hypoxia and oxidative stress-responsive gene, we further tested the effect of its exogenous expression on cell sensitivity to these types of stress. MCF-Hi95-2 and MCF-Hi95-20, as well as control cells, maintained either in the presence, or in the absence of tetracycline for 72 h were exposed to hypoxia/no glucose (no glucose, 5% CO_2, 0.5% O_2), or to 0.5–1 mM H_2O_2 for next 24 h. Both of the MCF7

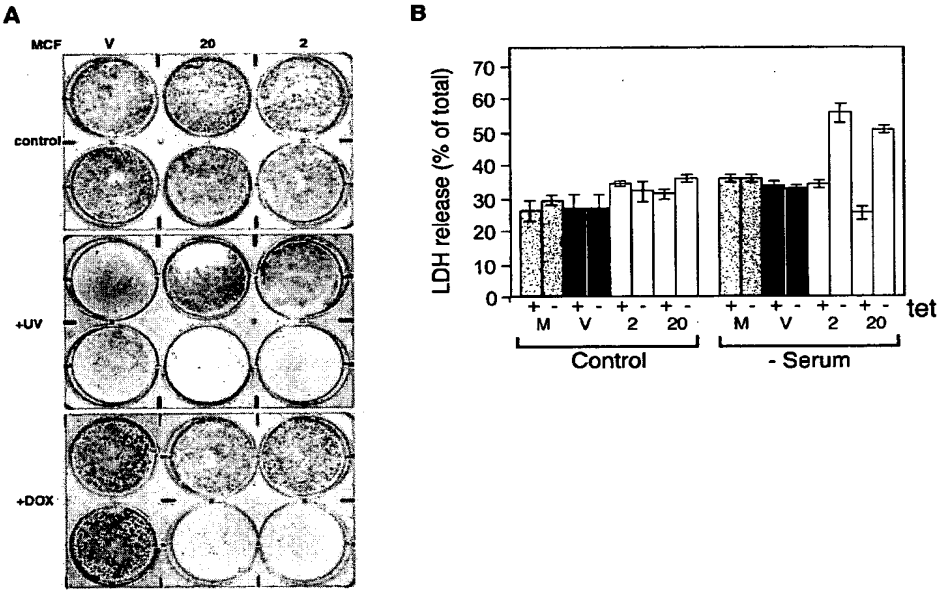

Figure 8 **(a)** Sensitization of MCF7 cells overexpressing Hi95 to doxorubicin and UV treatments. The photographs of methylene-blue stained cell culture plates containing either empty vector (V) transfected cells or inducible MCF7-tet-off Hi95 expressing cell clones, MCF-Hi95-2 (2) and MCF-Hi95-20 (20). The cells were maintained for 72 h either in the presence (three upper wells of each plate) or in the absence (three lower wells of each plate) of tetracycline. The cells were either left untreated (control) or were irradiated with UV for 1 min (+UV) or were treated with 100 ng/ml of doxorubicin for 4 days (+DOX). **(b)** Sensitization of MCF7 cells overexpressing Hi95 to serum starvation. M, parental MCF7-tet-off cells; V, MCF7-tet-off cells transfected with the empty pTet-Splice vector; 2, MCF-Hi95-2 cell clone; and 20, MCF-Hi95-20 cell clone. The cells were cultured either in the presence (+) or in the absence (−) of tetracycline and either under normal (control) or low serum (−serum) conditions. For details, see text and Materials and methods. Data represent the means of three independent experiments performed in triplicates

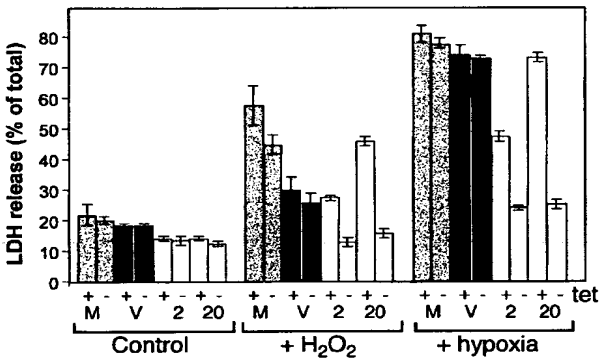

Figure 9 Protective effect of Hi95 overexpression on MCF7-tet-off cells cultured either under oxidative stress or hypoxia/no glucose conditions. M, parental MCF7-tet-off cells; V, MCF7-tet-off cells transfected with the empty pTet-Splice vector; 2, MCF-Hi95-2 cell clone; and 20, MCF-Hi95-20 cell clone. The cells were cultured either in the presence (+) or in the absence (−) of tetracycline and either under normal (control) or oxidative stress (H_2O_2) or hypoxia/no glucose (hypoxia) ischemia-mimicking conditions. For details, see test and Materials and methods. Data represent the means of three independent experiments performed in triplicates

cell clones expressing exogenous Hi95 were unexpectedly protected from apoptosis induced by hypoxia/no glucose or H_2O_2 treatment as was evident by microscopic observation, by measuring LDH release (Figure 9) and by Neutral Red uptake (not shown).

Discussion

Mammals respond to hypoxia by a series of adaptive responses that are controlled by activation of multiple genes (Bunn and Poyton, 1996). Products of these genes control the metabolic switch to glycolysis, increase oxygen delivery to tissues by stimulation of angiogenesis and erythropoiesis, and enhance cellular survival. The expression of most of the genes regulating the adaptive response to hypoxia is HIF-1-dependent (Dong *et al.*, 2001; Semenza, 1999). However, after prolonged and severe hypoxia, for example in such acute pathologies as stroke and myocardial infarction, the above adaptive responses may not be sufficient, and as a result cell death processes are initiated. Apparently, the decision regarding onset and particular type of cell death depends on the balance between different anti- and pro-apoptotic factors in a particular cell context. Hypoxia is capable of changing this balance through the activation and repression of pro- and anti-apoptotic genes via both HIF-1-dependent and independent mechanisms. Among anti-apoptotic genes, IGF2 (Feldser *et al.*, 1999) and IAP2 (Dong *et al.*, 2001) are induced by hypoxia through HIF1-dependent

and HIF1-independent mechanisms, respectively. Among genes involved in hypoxia-mediated apoptosis are HIF-1 independent p53 (An *et al.*, 1998; Graeber *et al.*, 1994, 1996) and Gadd153 (Price and Calderwood, 1992; McCullough *et al.*, 2001) as well as HIF-1-dependent genes Nip3 (Bruick, 2000; Vande Velde *et al.*, 2000; Sowter *et al.*, 2001) and a recently discovered RTP801 (Shoshani *et al.*, 2002). Apparently, other yet non-described genes may be involved in the regulation of cell death and survival in response to severe oxygen deprivation. Identification of these genes is important as they may represent valuable targets for the development of drugs modulating the outcome of acute ischemic diseases.

We anticipated that expression of proapoptotic genes should be connected to the decompensation phase of hypoxia response and thus be delayed in time in regard to stimulation. Several dozens of such genes (not reactive at early time points following oxygen deprivation) were identified employing microarray analysis of gene expression in A172 cells exposed to hypoxia for 16 h. One of the previously unknown genes designated Hi95 was chosen for further analysis, mainly due to its close homology to a p53-regulated gene PA26, previously described as a member of the GADD family (Velasco-Miguel *et al.*, 1999). Late induction of Hi95 was observed also *in vivo* in rat brains subjected to middle cerebral artery occlusion (MCA). PA26, Hi95 and one additional hypothetical protein comprise a novel protein family and obtained the names of sestrin 1, 2 and 3, respectively. This family is unlikely to originate from recent direct gene duplication in mammalian genome, since the search of Ensemble database has revealed that genes corresponding to sestrins are localized to three different human chromosomes: PA26 (sestrin 1) – to 6p21 (Velasco-Miguel *et al.*, 1999)); Hi95 (sestrin 2) – to 1p35.3, and sestrin 3 – to 11q21.

The induction of Hi95 mRNA by hypoxia (DFO treatment) was reproduced in several cell lines of different origin. It was proven to be independent of the p53 status of cells as could have been also anticipated from the presence of missense mutation in codon 242 of p53 in A172 cells used for gene discovery (Kataoka *et al.*, 2000). On the contrary, expression of PA26 was stimulated by hypoxia only in cells possessing wt p53 protein (i.e. MCF7, LIM1215). Only the 2.6 kb p53-dependent transcript (Velasco-Miguel *et al.*, 1999) appeared induced, implying the regulation through the p53-responsive promoter element (Graeber *et al.*, 1994). Though we do not have direct proof, the indirect evidence supports the conclusion that regulation of Hi95 by hypoxia is independent also of HIF-1α, a transcription factor known to regulate expression of numerous genes in hypoxic conditions (Semenza, 2000). First, the nucleotide sequence analysis of 2 kb upstream sequences as well as of introns within the Hi95 gene did not reveal any potential strong HIF1α binding sites. Second, kinetics of its hypoxia response mostly resembles the one of HIF-1α-independent gene GADD153 and differs from HIF1α-dependent genes p21-Waf1 and VEGF (Carmeliet *et al.*, 1998; Liu *et al.*, 1995). Third, our preliminary results demonstrate that in the VHL-deficient 786-0 renal carcinoma cell line with constitutively activated HIF-1α (Stratmann *et al.*, 1997), expression of Hi95 is low and is dramatically stimulated following 24 h of DFO treatment (A Budanov and PM Chumakov, unpublished observation), contrasting the constitutive expression of HIF-1α-dependent genes.

The factors important for hypoxia-dependent expression of Hi95 are yet to be discovered. Analysis of Hi95 expression pattern points out at certain peculiarities of Hi95 response to hypoxia in different cell lines. Thus, Hi95 was not induced by DFO treatment of LIM1215, HeLa and HT1080 cells though other genes, both HIF-1α-dependent and independent, were induced. The observed phenomenon did not depend on the basal levels of Hi95 expression, which significantly differ in these three cell lines. Interestingly, in addition to Hi95, DFO also failed to stimulate expression of PA26 in HeLa and HT1080 cells. In general, hypoxia response to PA26 was not observed in p53-negative cells (HeLa, MDAH041, NCI-H1299), but this particular regulation is likely to be independent of p53, since HT1080 cells are p53-positive. Altogether, the data suggest the existence of certain common unique pathway elements responsible for Hi95 and PA26 hypoxic stimulation. The same elements are likely to participate in the response of both genes (but not waf1) to DNA damage since it also appeared blocked in HT1080 cells.

The data, however, strongly indicate that the major regulator of transcriptional response of Hi95 to DNA damage is p53 (similar to regulation of PA26), since expression of Hi95 was affected by genotoxic treatments exclusively in cell lines expressing wild-type p53 (MCF7, LIM1215, NCI-H1299-tet-wt53). The kinetics of Hi95 response to DNA damage coincides with that of p21-waf1, a well-known transcriptional target of p53. Moreover, the nucleotide sequence analysis of the Hi95 gene revealed two potential p53-binding sites localized in the upstream region and in intron 1 (A Budanov and PM Chumakov, unpublished observation). Altogether, the data suggests that Hi95 is a novel p53-target gene. Among other stresses tested, only the oxidative one (treatment with H_2O_2) triggered the Hi95 transcription whereas both heat shock and serum starvation had no effect in all cell lines tested regardless of their p53 status.

We concluded that, since the Hi95 gene is induced in response to a variety of stressful conditions, it may be regarded as a typical member of GADD family of genes, involved in regulation of cell growth and survival (Batchvarova *et al.*, 1995; Takekawa and Saito, 1998; Zhan *et al.*, 1996). Consistent with this conclusion, we found that Hi95 may be classified as GADD gene not only because of the mode of its regulation but also because of its functional properties – capability to negatively regulate cell growth under certain conditions. This functional feature was confirmed by a number of observations like i.e.: (1)

apoptosis of HEK293 cells transiently transfected with Hi95; (2) a reduced colony formation ability of several types of Hi95 transfected cells (regardless of their p53 status); (3) a rapid disappearance of exogenous Hi95 expressing cells from mass cultures of transfected MCF7 cells; (4) hypersensitivity of MCF7-tet-Hi95 cells to doxorubicine, UV irradiation and serum starvation. Interestingly, in contrast to DNA-damaging drugs, treatment with taxol revealed no Hi95-dependent difference in the viability of MCF7 cells, implying a certain specificity in the ability of Hi95 to sensitize cells to various treatments. The proapoptotic activity of Hi95 starts to be evident approximately 24 h following induction of its exogenous expression as detected both in transient transfection experiments and in experiments employing tetracycline-repressible cell clones. The experimental data is consistent with the picture observed in ischemic brain regions following MCAO: while at 12 h post stroke Hi95 expressing neurons looked normal, at 24 h they displayed the signs of damage (chromatin condensation, shrinkage etc).

Paradoxically, the effect of Hi95 gene expression on cells cultured under ischemia-mimicing conditions (no glucose and 0.5% O_2) or under oxidative stress (H_2O_2) was different from that observed with DNA-damaging drugs, namely protective. Taking into account the above-mentioned correlation between Hi95 expression in post-stroke rat brain with neuronal death, it is currently unclear how to interpret the potential contradiction between the results obtained under hypoxic conditions *in vitro* and *in vivo* in regard to Hi95 function in hypoxia. However, it is worth mentioning that RTP801, a novel HIF-1α-responsive gene recently identified by us, showed a similar pattern of behavior: protecting continuously growing cells in culture and being detrimental for resting cells *in vivo* (Shoshani *et al*., 2002).

Nothing is known about the biological function of either sestrin 1 or sestrin 3. However, a close structural similarity of all three sestrins suggests the accompanying functional similarity. Thus, the differences in their responsiveness to stress (e.g. hypoxia induction of Hi95 but not of PA26 in p53-negative cells, or induction by serum starvation of PA26 but not of Hi95 gene) may be compensated by their potential functional redundancy.

Further identification of molecular pathways targeted by Hi95 gene and by other members of the sestrin gene family might open new approaches for modulation of cell responses to stressful conditions, leading to development of novel strategies for therapeutic interventions.

Materials and methods

Cell lines and treatment conditions

The *Human glioblastoma cell line A172* (Pinkerton and Rana, 1976) (p53 codon 242 Cys to Phe mutation (Kataoka *et al*., 2000)) was maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 20 U/ml penicillin, and 20 μg/ml streptomycin. Hypoxia in these cells was achieved by maintaining them in a three-gas incubator in an atmosphere of 0.5% O_2 and 5% CO_2 for either 4 or 16 h.

Human lung carcinoma cell line NCI-H1299 (p53-null), *human skin fibroblasts MDAH041 from a Li-Fraumeni patient* (Bischoff *et al*., 1991) (p53-null), *human ovary carcinoma cell line SKOV-3* (Dini *et al*., 1980) p53-null), *human breast carcinoma cell line MCF7* (Westley and Rochefort, 1979) (wild-type p53), *HEK293* (human embryonic kidney cells transformed with adenovirus type 5 early region DNA fragment (Graham *et al*., 1977)), human fibrosarcoma cell line HT1080 (Jones *et al*., 1975) (wild type p53), and *human colon carcinoma cell line LIM1215* (Whitehead *et al*., 1985) (wild type p53) were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 20 U/ml penicillin, and 20 μg/ml streptomycin. *MCF7 Tet-off* (Clontech), *NCI-H1299-tet-off cell strains* (Clontech) carrying tetracycline-dependent transcriptional regulator tTA (Gossen and Bujard, 1992) and their transfected derivatives were maintained in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 20 U/ml penicillin, 20 μg/ml streptomycin, and 100 μg/ml neomycin. The transfectants and vector-bearing control cells were cultured in the presence of 1 μg/ml tetracycline and 100 μg/ml hygromycin. To induce exogenous gene expression, the cells were washed with PBS, plated and incubated in tetracycline-free medium for 72 h.

The cells were usually split 24 h prior to treatment. For induction of hypoxia, iron chelator deferoxamine mesylate (DFO) (Sigma) was added to the culture medium at a concentration of 300 ng/ml for 24 h. To measure levels of gene expression following the genotoxic stresses, the cells were treated either with 200 ng/ml of doxorubicin for 24 h, or with UV (Model 3-UV transilluminator, UVP, Apland, CA, USA, 302 nM, 2500 $\mu W/cm^2$, 23 s) or with 10 Gy γ-irradiation. Oxidative stress was achieved by incubation of cells with 0.5–1 mM H_2O_2 for 24 h. Hyperthermia was achieved by incubation of cells at 44°C for 15 min followed by 24-h incubation at 37°C prior to RNA isolation. Incubation in low-serum medium (0.1%) was performed for 24 h.

Plasmids

pcDNA3-Hi95-Flag construct was prepared by subcloning of a complete ORF of human Hi95 cDNA (nucleotides 316–1764) derived from the λ phage clone into the *Hind*III/*Not*I sites of the pcDNA3 expression plasmid (Invitrogen). The cDNA fragment was obtained by PCR amplification on the λ phage template using human Hi95-specific primers containing corresponding endonuclease restriction sites and Flag-tag sequence. Forward primer: 5′-AAAAGCTTGCCACCATG-GATTACAAGGACGACGACGATAAGATGAT CGTGG-CGGA-3′; reverse primer: 5′-AAGCGGCCGCTCAGGT-CATGTAGCGGGGA-3′.

Tetracycline-repressible pTet-Splice Flag-Hi95 construct was prepared by subcloning the *Hind*III–*Not*I/blunt insert from pcDNA3-Hi95-Flag into *Hind*III–*Eco*RV sites of pTet-Splice (Invitrogen).

The pBS-ratHi95 construct used for the preparation of riboprobes for *in situ* hybridization contained a 496 bp rat Hi95 cDNA fragment corresponding to the 5′ region of Hi95 ORF (nucleotides 359–854), inserted into *Eco*RV site of pBS vector. A Rat-specific probe was obtained by RT–PCR with the degenerative primers designed on the basis of human sequence.

Northern blot analysis

Total cellular RNA was extracted with Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. A 10 μg aliquot of each RNA sample was electrophoresed through 1% agarose-formaldehyde gel, transferred to HybondN nylon membrane (Amersham) and fixed by heating at 80°C for 2 h in the vacuum oven. The hybridization conditions were as described (Sambrook *et al.*, 1989). The cDNA probes for hybridizations were obtained by RT–PCR on total polyA RNA template from primary human embryo fibroblasts. The following primers were used to obtain cDNA fragments corresponding to protein coding regions of the following genes: human p21-WAF1 cDNA (503 bp), 5'-primer: 5'-GGCGCCATGTCAGAACCGGCTGG; 3'-primer: 5'-GATTAGGGCTTCCTCTTGGAGAAG; human VEGF cDNA (509 bp), 5'-primer: 5'-TCCGAAACCATGAACTTTCTGCTG; 3'-primer: 5'-CCGGCTCACCGCCTCGGCTTGT; human GADD153 cDNA (731 bp), 5'-primer: 5'-TTCCAGACTGATCCAACTGCAGAG; 3'-primer: 5'-TCACTTTAATAGATAGGGACAGTC; human PA26 cDNA (448 bp), 5'-primer: 5'-CTTGGCATTAGAATTCCTCGACCAC; 3'-primer: 5'-CCTCAATGTGTTCTTTGGTAATAAG; human Hi95-specific probe was derived as a 501 bp cDNA fragment from the 5'-part of Hi95 cDNA, showing minimal homology to PA26. 5'-primer: 5'-TGAAAACCCCTGCGCTGCG; and 3'-primer: 5'-CATCACCACTGCCAGGTTG.

^{32}P radioactive probe labeling was performed using Multiprime Random Priming Labeling kit (Amersham).

Western blot analysis

Whole cell protein extracts from MCF7-tet-off cells and their derivatives were prepared in RIPA lysis buffer (RIPA buffer: 50 mM tris-HCL pH 7.4; 150 mM NaCl; 1% deoxycholate Na; 1% NP-40; 0.1% SDS, 100 μM PMSF; 1 μM pepstatin A; 1 μM E64). The extracts were normalized for protein content, resolved on a 4–20% gradient polyacrylamide-SDS gel (Novex) and transferred to HybondP membrane (Amersham). The uniformity of protein loading on the gel was verified by subsequent Ponceau S staining of the membrane. The membrane was then blocked in PBS containing 10% milk and 0.1% Tween20 for 1 h at 23°C and incubated with anti-Flag monoclonal antibodies M2 (SIGMA) at a concentration of 10 μg/ml for a further hour at 23°C in the same buffer. After washing, the membrane was incubated for 1 h at 23°C with the second antibody (anti-mouse IgG 0.2 μg/ml, Santa Cruz) conjugated to horseradish peroxidase. The blots were processed using ECL-Plus Reagents (Amersham) according to the manufacturer's instructions.

Preparation of probes and hybridization to cDNA microarray

Total RNA derived from A172 glioblastoma cells maintained either under normal (control) or hypoxia conditions (4 or 16 h) was used for the preparation of hybridization probes. Labeling of probes for microarray hybridization was performed by reverse transcription on 50 μg of total cellular RNA template and oligo-dT_{18} as a primer. RNAs from control and hypoxia-treated cells were labeled with Cy3-dCTP and Cy5-dCTP (Amersham), respectively. The following sets of probes were utilized for microarray hybridizations: total control RNA (Cy3) and total RNA from 4 h hypoxia (Cy 5); total control RNA (Cy 3) and total RNA from 16 h hypoxia (Cy 5). The probes were hybridized to UniGEM1 microarray (Incyte). Hybridization, washing and scanning of the slides were performed as previously described (Schena *et al.*, 1996).

Transient transfection of HEK293 cells

1×10^6 HEK293 cells grown in 100-mm dishes were transfected with 10 μg of plasmid DNA using the calcium phosphate transfection method as previously described (Chen and Okayama, 1987).

Preparation of MCF7 cells with inducible Hi95 expression

2×10^5 MCF7 Tet-off cells (Clontech) were co-transfected with 2.5 μg of pTet-Splice Flag-Hi95 (or control empty) and 0.5 μg of pTK-Hyg (Clontech) vector DNA using Lipofectamine Reagent (Invitrogen). The cells were then grown in selection medium containing 100 μg/ml of hygromycin (Roche) and 1 μg/ml of doxicycline (Sigma).

Preparation of NCI-H1299 cells with inducible wt p53 expression

A sub-line of human lung carcinoma cells NCI-H1299 devoid of endogenous p53 expression and bearing tetracycline regulator tTA (Gossen and Bujard, 1992) was infected with self-inactivation retroviral construct pSIT-wt-p53-neo (Alexandrova *et al.*, 2000) that contains human wild-type p53 cDNA under the control of tetracycline-regulated promoter. The cells were grown in the presence of 500 μg/ml of G418 (SIGMA) and 1 μg/ml of tetracycline (Sigma). One of the G418 resistant clones showing 25–50-fold induction of wild type p53 expression upon removal of tetracycline from the culture medium was used for further studies.

Colony formation assay

2×10^5 of cells (HT1080, NCI-H1299, MCF7, MDAH041 and LIM1215 cell lines) were plated in 6 well dishes and grown for 24 h. The medium was then replaced with fresh serum-free DMEM. One μg of pcDNA3-Hi95, or control pcDNA3, or pcDNA3-wt-p53 DNA were transfected along with 0.1 μg of EGFP-expressing vector DNA pEGFP-N1 (Clontech) DNA using Lipofectamin-Plus Reagent (Invitrogen). The cell numbers subjected to drug selection were normalized according to the numbers of GFP-positive cells. The G418 (500 μg/ml) selection continued for 10–14 days, after which the cells were fixed on the plates and stained with methylene blue.

Assessment of sensitivity of Hi95 expressing MCF7 cells to various stresses

1×10^4 cells of MCF-Hi95-2, MCF-Hi95-20 and MCF7-vector-7 clones were plated in 6-well dishes and grown for 72 h either in the presence or in the absence of 1 μg/ml tetracycline. For UV treatment, the cells were irradiated with 100 Mj/cm^2 short wavelength UV for 1 min (UV Crosslinker, Fisher) and then incubated at 37°C for 24 h. Alternatively, the cells were treated with either doxorubicin or with taxol at 100 ng/ml for 4–5 days. The cells were then fixed and stained with methylene blue. To test the sensitivity of cells to serum starvation, the MCF7-tet-off cell clones were plated at a density of 10^4 cells in a 6-well dishes in DMEM containing 10% FBS with or without 1 μg/ml of tetracycline. The medium was replaced 72 h

later with fresh medium containing 0.1% FBS with or without tetracycline. Twenty-four hours later, the cells viability was assessed by LDH leakage and by Neutral Red assays.

Methylene blue staining of adherent cells

The cells on the plates were washed three times with PBS, fixed with 100% methanol for 10 min at room temperature, stained with 1% methylene blue in PBS, washed in the running water and dried. For quantitative estimations the dye was extracted with 1% SDS, and optical density was measured at 640 nM.

Cell viability assays

Annexin V staining of apoptotic cells was performed using the Annexin V-Cy3 Apoptosis Detection kit (BIOVISION) according to the manufacturer's protocol.

Neutral red assay was conducted as described before (Wallach, 1984), using the Neutral Red dye (Sigma). All measurements were performed in triplicate at optical density at 540 nM.

LDH leakage assay was performed using a Cytotoxicity Detection Kit (Molecular Biochemicals) according to the manufacturer's protocol. LDH activity was measured at optical density of 492 nM. LDH release was defined as the ratio of LDH activity in the medium to LDH activity corresponding to total cell death (according to the manufacturer's instructions) and was expressed in percentage. All measurements were performed in triplicate.

In histological sections, apoptotic cells were detected by DNA end labeling assay (TUNEL). The assay was performed using ApopTag® Peroxidase *In Situ* Apoptosis Detection kit (Intergen Company, NY, USA) according to manufacturer's protocol.

Permanent middle cerebral artery occlusion (MCAO) stroke model

The stroke model was prepared in a spontaneously hypertensive rat strain (SHR) as previously described (Leker *et al.*, 2001). Experimental animals were sacrificed 0.5, 1, 2, 12, 24, 48 and 72 h after the operation (two animals per time point). The brains were removed, fixed in formalin, embedded in paraffin, and coronal sections were performed for further use in *in situ* hybridization with ^{35}S-UTP labeled rHi95- and rVEGF-specific sense and antisense riboprobes.

In situ *hybridization*

Rat Hi95 radioactive riboprobes were produced from the pBluescript-rHi95 vectors (see above) using either T7-RNA polymerasse (antisense probe) or T3-RNA polymerase (sense probe) as previously described (Komarova *et al.*, 2000). *In situ* hybridization was performed according to a previously published protocol (Faerman and Shani, 1997). The exposed slides were developed in Kodak D-19 developer, fixed in Kodak fixer and counterstained with hematoxilin-eosin. The microphotographs were taken using Zeiss Axioscop-2 microscope equipped with the Spot RT CCD camera (Diagnostic Instruments).

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